Virology **259,** 219–227 (1999)
Article ID viro.1999.9768, available online at http://www.idealibrary.com on IDEAL®

Carboxy-Terminal Five Amino Acids of the Nucleocapsid Protein of Vesicular Stomatitis Virus Are Required for Encapsidation and Replication of Genome RNA

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Received February 16, 1999; returned to author for revision March 17, 1999; accepted April 20, 1999

The encapsidation of vesicular stomatitis virus (VSV) genome RNA, a prerequisite step to the replication process by the nucleocapsid protein (N) was studied by its ability to package VSV leader RNA *in vitro* in a RNase-resistant form. The VSV leader RNA was derived from the SP6 transcription vector while the N protein was made in rabbit reticulocyte lysate. The *in vitro* encapsidation was carried out by translating N mRNA in the presence of ^{32}P-labeled presynthesized leader RNA. The RNA encapsidation property of the N protein was completely abrogated when the C-terminal five amino acids (VEFDK-COOH) were deleted. Systematic mutational analyses within the C-terminal five amino acid regions reveal that the RNA encapsidation activity was lost in all mutants except K → A and K → R, indicating that C-terminal five amino acids, in particular the lysine residue play critical role in genome RNA encapsidation. To correlate the *in vitro* encapsidation abilities of these mutant N proteins with genome RNA replication, we have used a full-length cDNA clone of VSV genome RNA to rescue infectious virions from cells expressing L, P, and wt or mutant N proteins and measured the recovery of plaque forming units. The results indicate that the N mutants that are defective in *in vitro* encapsidation of leader RNA do not support replication, establishing the requirement of C-terminal five amino acids of the N protein in viral replication.

INTRODUCTION

Vesicular stomatitis virus (VSV), a negative-strand RNA virus, remains one of the best-studied animal RNA viruses. This is primarily due to the availability of both *in vitro* (Banerjee and Barik, 1992; Das *et al.,* 1996) as well as *in vivo* (Pattnaik *et al.,* 1992; Lawson *et al.,* 1995; Stillman *et al.,* 1995; Whelan *et al.,* 1995) assay systems for studying viral transcription and replication processes. It has been well documented that three virus-encoded proteins are required for transcription and replication of the genome RNA (Emerson and Yu, 1975; Banerjee, 1987; Banerjee and Barik, 1992); these include the nucleocapsid protein N (49 kDa), which enwraps the genome RNA in an ordered helical structure that serves as the template for transcription and replication; the phosphoprotein P (30 kDa), a subunit of RNA polymerase that functions as a transcription factor; and the RNA-dependent RNA polymerase L (240 kDa). In infected cells, the nucleocapsid core that consists of N-RNA complex, the resident P protein, and the L protein initiate transcription to generate subgenomic mRNAs, which are translated to produce viral proteins required for replication of the genome RNA. During the replicative phase, first a full-length complement (antigenome or positive sense) of genomic RNA is synthesized in the form of a nucleocapsid, which in turn acts as the template for synthesis of progeny negative sense genome-containing nucleocapsid. Although it is generally believed that the same polymerase performs both transcription and replication processes, our recent findings, however, strongly suggest that the transcriptase and replicase are composed of two distinct complexes containing L-P_{2-3} (where P is either dimer or trimer) and L-(N-P), respectively (Das *et al.,* 1997). During replication of the genome RNA, a soluble form of the N protein is required (Wertz *et al.,* 1987). This form of the N protein has been proposed to interact with nascently transcribed RNA chains from the RNP complex and encapsidate the newly synthesized genome RNA in a tightly assembled structure. This results in the formation of a N-RNA complex containing full-length plus- or minus-strand genome RNA. The RNA within the newly formed N-RNA complex is totally resistant to RNase digestion. Thus the interaction and subsequent encapsidation of the plus- and the minus-strand genome RNAs with the N protein is the fundamental step in the replicative cycle of the virus. It has been suggested that this interaction of the N protein with the nascent RNAs occurs within the leader sequence during its synthesis from nucleocapsids containing either the positive- or the negative-strand genome RNA (Blumberg *et al.,* 1983; Giorgi *et al.,* 1983). Thus the positive or negative leader RNAs presumably encode the recognition signal for en-

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0042-6822/99 $30.00

capsidation by the N protein. Direct evidence that the N protein indeed interacts *in vivo* with the leader RNA came from the observation that N protein leader RNA complexes are produced in infected cells that are RNase resistant. Encapsidation by N protein appears to initiate within the first 14 nucleotides of nascent leader RNA, which contains A residues repeated at every third base (Giorgi *et al.,* 1983; Nichol and Holland, 1987).

In addition to the N protein, specific roles played by the phosphoprotein P in the replication process have also been documented (Peluso and Moyer, 1988; Howard and Wertz, 1989; La Ferla and Peluso, 1989). The *in vitro* studies suggest that the N protein alone can initiate replication, but the presence of P protein greatly stimulated the replication reaction. Moreover it was demonstrated both *in vitro* and *in vivo* that N and P proteins form specific complexes (Masters and Banerjee, 1988a; Howard and Wertz, 1989; La Ferla and Peluso, 1989; Takacs *et al.,* 1993; Takacs and Banerjee, 1995). However, the mechanistic role of these N–P complexes in the encapsidation process remains an enigma. It has been proposed that the P protein not only interacts with the N protein to keep the latter protein in a replication-competent state but may also impart specificity to the N protein to encapsidate the VSV genomic RNAs (Masters and Banerjee, 1988b; Peluso and Moyer, 1988; Howard and Wertz, 1989; Das and Banerjee, 1992). The C-terminal end of both N and P proteins appears to be involved in this interaction process (Takacs *et al.,* 1993; Takacs and Banerjee, 1995). Using the two-hybrid system, we have demonstrated that the C-terminal 10 amino acids of the P protein and C-terminal 5 amino acids of the N protein are critical for such N–P interaction (Takacs *et al.,* 1993; Takacs and Banerjee, 1995).

The regulatory roles of *cis*-acting elements in the genome RNA during viral replication process have been extensively studied using recently developed reverse genetic system (Wertz *et al.,* 1994; Stillman *et al.,* 1995; Li and Pattnaik, 1997). This system has been used successfully for VSV to produce genomic or DI RNAs that would transcribe or replicate, respectively, in cells in the presence of cDNA-encoded viral proteins (Pattnaik *et al.,* 1992; Lawson *et al.,* 1995; Stillman *et al.,* 1995; Whelen *et al.,* 1995). We have recently shown using VSV minireplicon, that the P mutant that is defective in forming complex with N is incapable of supporting replication *in vivo* (Das *et al.,* 1997). These findings supported the notion that while transcriptase is composed of L-P_{2-3} (where P_{2-3} is a dimer or trimer) the replicase appears to be a tripartite complex consisting of L-[N–P], which initiates the replication reaction.

So far, no specific domain(s) within the N protein has been identified for its genome RNA encapsidation activity. To map the encapsidation signal within the N protein, we have developed both *in vitro* and *in vivo* assay systems directed toward the encapsidation of VSV genome RNA by the N protein. A series of deletion and substitution mutants of N_{NJ} protein has been generated and analyzed for its ability to support encapsidation of VSV_{NJ} leader RNA *in vitro* and replication of genome RNA *in vivo* using cDNA clone of VSV using a reverse genetic system. The results presented in this communication indicate that C-terminal five amino acids of the N protein, in particular the penultimate Lysine residue, plays an important role in the encapsidation/or replication of VSV genome RNA.

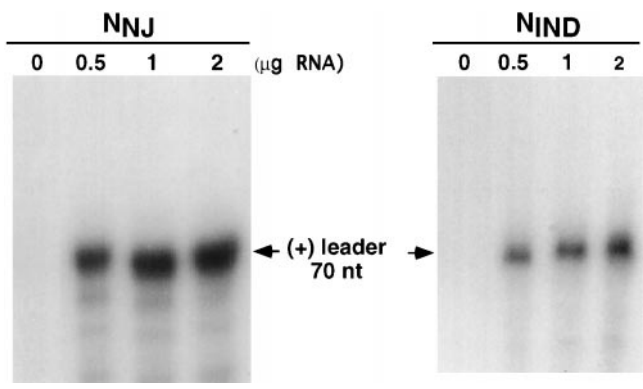

FIG. 1. *In vitro* encapsidation of (+)-leader RNA of VSV_{NJ} by homologous and heterologous N proteins. Increasing amounts of N mRNA of either serotype were translated in 30 μl rabbit reticulocyte lysate in the presence of 0.1 μg ^{32}P-labeled leader RNA (~100,000 cpm). After translation, the samples were treated with RNaseA at a final concentration of 25 μg/ml at 37°C for 30 min. Protected leader RNA was analyzed on a 10% polyacrylamide gel containing 8 M urea. Migration position of 70-nt-long leader RNA is shown by arrows.

RESULTS

The role of the C-terminal end of the N protein in the encapsidation of leader RNA *in vitro*

We have previously shown (Das and Banerjee, 1992, 1993) that *in vitro* as well as bacterially made VSV N protein specifically encapsidated leader RNA over non-leader RNA, rendering the former resistant to RNaseA digestion. To put our experimental approach in proper perspective, we repeated the *in vitro* experiments involving the encapsidation of leader RNA by heterologous N protein. ^{32}P-labeled VSV_{NJ} leader RNA was synthesized by SP6 polymerase from a pUC 19 vector containing SP6 promoter fused with an insert composed of 47 nucleotides leader sequence (5′ sequence of the antigenomic RNA) up to AUG of the N gene (a total 70 nt). As shown in Fig. 1 by RNaseA protection experiments, the ^{32}P-labeled VSV_{NJ} leader RNA was encapsidated *in vitro* by the N protein of either NJ or IND serotype; the encapsidation appeared to be more efficient by the N protein of the same serotype (5–6% of input leader RNA) than the heterologous N (1–2%). Similar observations were also reported by other laboratories (Smallwood *et al.,* 1994) where the N protein of either serotype was shown to

encapsidate heterologous leader RNAs. Thus taken together we can conclude that the encapsidation or RNA binding domain(s) within the N protein of either serotype are similar and recognize a similar cognate sequence in the leader RNA of either serotype (Das and Banerjee, 1992, 1993). To locate the putative domain(s) responsible for the encapsidation of the genome RNA within the N protein, we initially made a series of deletion mutants of N_{NJ} in both N and C termini with the help of polymerase chain reaction. For introducing specific mutations at the C-terminal end of the N protein, we amplified a 300-nucleotide-long DNA fragment of the N_{NJ} gene containing various mutations and replaced the PCR-amplified fragments into the wild-type N gene using unique *Xho*I and *Hin*dIII sites of pN109 (Masters and Banerjee, 1988a). To obtain deletion mutations at the N-terminal end, we amplified the entire N gene using upstream primers that contained a unique *Eco*RI site followed by respective deletion mutations and the complementary sequences to the 5′ end of the N gene. The downstream primer contained the complementary sequence to the 3′ end of the N gene followed by a *Hin*dIII site. The PCR-amplified products were then introduced into pGEM-4 vector at the *Eco*RI and *Hin*dIII sites. By these procedures, we created deletion mutants of the N gene corresponding to 5, 10, or 15 amino acid deletions from the C-terminal end and 5 or 10 amino acid deletions from the N-terminal end (Fig. 2A).

The encapsidation activity of the N_{NJ} mutants was measured with respect of their abilities to encapsidate VSV_{NJ} leader RNA *in vitro*, in a RNase-resistant form. Results of the RNaseA protection experiments by N mutants are shown in Fig. 2B. It can be seen that deletion of as few as five amino acids from the C-terminal end of the N protein totally abrogated the encapsidation of leader RNA by the N protein. In contrast, truncation of 10 amino acids from the N-terminal end had very little effect on its RNA encapsidation activity. These results suggest that the encapsidation domain of the N protein probably resides within the C-terminal five amino acids. To confirm that all mutant N mRNAs used in the above experiment were efficiently translated in the cell-free system, the ^{35}S-labeled N proteins were analyzed by 10% SDS–PAGE. As shown in Fig. 2C, each mutant N protein was synthesized in comparable amount during the encapsidation reaction.

We have previously shown that the wt N protein synthesized *in vitro* enters into the nondenaturing gel when the nascent protein binds to the endogenous RNA present in the rabbit reticulocyte lysate (Masters and Banerjee, 1988b). To test how the above deletion mutants of the N protein migrate in such gel system, we performed *in vitro* translation of the mutant mRNAs in rabbit reticulocyte lysate and analyzed the products in a 7.5% nondenaturing polyacrylamide gel. Unlike wild-type or N-terminal mutants, none of the C-terminal truncated N proteins entered into the 7.5% nondenaturing gel, confirming that the C-terminal deletion mutants lacked RNA binding property (data not shown).

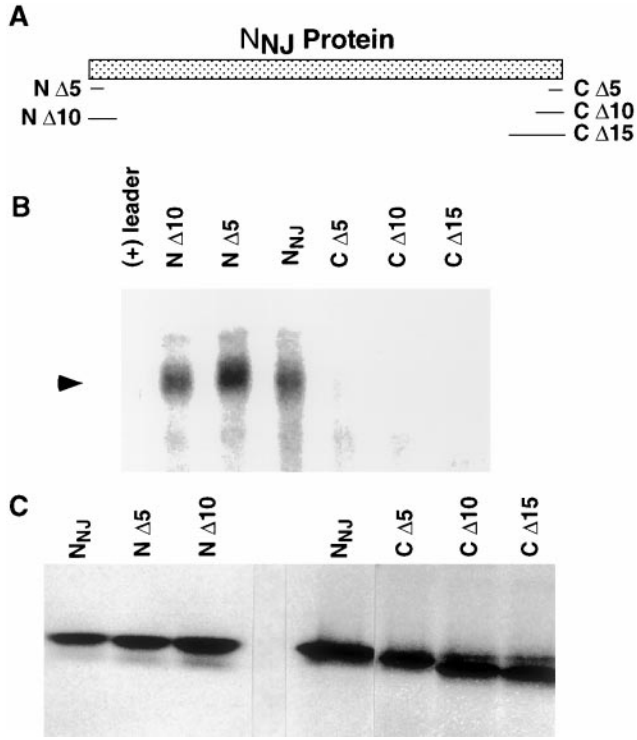

FIG. 2. Ability of truncated N_{NJ} proteins to encapsidate VSV leader RNA. (A) Schematic diagram of N protein showing progressive deletion mutants from both termini. The deleted N genes were cloned into pGEM4 vector under SP6 polymerase. CΔ5, CΔ10, CΔ15 indicate deletion of 5, 10, and 15 amino acids, respectively, from C-terminal end, whereas NΔ5, NΔ10 indicate 5 and 10 amino acids, respectively, from N-terminal end. (B) Similar RNaseA protection assay as shown in Fig. 1 was performed by incubating 0.5 μg of each N mRNA with 0.1 μg ^{32}P-labeled leader RNA (~100,000 cpm). Migration position of leader RNA (70 nt) is shown by the arrow. (C) Flurogram of ^{35}S-labeled protein profile of mutant N proteins.

It is important to note that there is a high degree of amino acid sequence similarity (>80%) between the N proteins of both NJ and IND serotypes of VSV (Banerjee *et al.,* 1984). Moreover, if one aligns the C-terminal 10 amino acid sequences of N_{NJ} and N_{IND}, the sequences are virtually identical to only one nonidentical change (S → V) (Fig. 3A). Because the deletion of C-terminal five amino acids completely abolished the encapsidation property of the N protein, we were interested in further investigating the role of individual amino acid within this region in the encapsidation of the leader RNA *in vitro*. We took two approaches for mutational analysis of this region: (1) we removed one amino acid at a time from the C-terminal end and (2) we introduced nonconserved amino acids in the region by site-directed mutagenesis. A battery of N mutants were constructed, and their encapsidation activities were examined as described above and shown in Fig. 3B. Surprisingly, deletion of the

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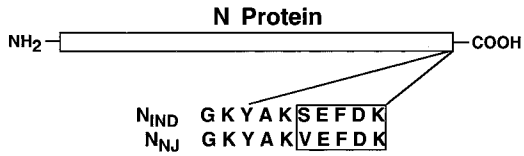

B

Mutant N proteins	Leader encapsidation
WT	+
C ΔK	-
K -> R	+
K -> A	+
K -> D	-
K -> I	-
DK -> AA	-
VEF -> AAA	-
VEFDK -> A_5	-

FIG. 3. Effect of mutation within the C-terminal five amino acids of N_{NJ} protein on its encapsidation ability. (A) Alignment of C-terminal amino acid residues of the N proteins of both NJ and IND serotypes. (B) RNaseA protection analysis of leader RNA was carried out by translating mutant N mRNAs in rabbit reticulocyte lysate containing ^{32}P-labeled leader RNA as described in Fig. 2.

terminal Lys residue totally abrogated the encapsidation property of the N protein. All other mutations altering the K residue or changing VEFDK sequence abolished the encapsidation property except for two: K → R and K → A. The replacement of K either by R or A, perhaps, restored the structural integrity of the N protein necessary for its encapsidation activity. The above results suggest that C-terminal five amino acids of the N protein are directly involved in the encapsidation of VSV genome RNA and consequently in the replication process.

Rescue of VSV from cells expressing mutant N proteins

Because the encapsidation by N protein of the viral genome RNA is a prerequisite step to the replication process, it would be interesting to examine whether the mutant N proteins that are defective in *in vitro* encapsidation of the leader RNA are also defective in replication of genome RNA *in vivo*. So far, all the N mutants used in our *in vitro* studies were made in the N protein of NJ serotype. Because of the unavailability of a full-length L gene of VSV_{NJ}, the *in vivo* reverse genetic system has not yet been developed for New Jersey serotype. On the other hand, the recovery of infectious virus from a full-length cDNA clone of VSV_{IND} genome RNA has been well established that renders the biology of VSV Indiana serotype fully accessible to genetic manipulation (Lawson *et al.,* 1995; Whelan *et al.,* 1995). To use VSV_{IND} reverse genetic system for testing the abilities of N_{NJ} mutant proteins to support genome RNA replication *in vivo*, it was necessary first to establish whether the wild-type N_{NJ} could support encapsidation/replication of VSV_{IND} from cDNA in the presence of L_{IND} and P_{IND} proteins. On the basis of the fact that the N proteins of both NJ and IND serotypes are highly similar (>80%) in amino acid sequence (Banerjee *et al.,* 1984) coupled with the earlier findings that the N protein of either serotype can encapsidate heterologous leader RNA (Das and Banerjee, 1992, 1993; Smallwood *et al.,* 1994), it is conceivable that N_{NJ} would support rescue of infectious VSV_{IND} in the presence of L_{IND} and P_{IND}. In this regard, it is important to mention that VSV_{IND} DI replication has been observed in cells coinfected with the VSV_{IND} DI particle and wild-type VSV_{NJ}, indicating that the N protein of NJ serotype is able to encapsidate heterologous (IND serotype) genome RNA *in vivo*. Therefore we tested the ability of N_{NJ} to rescue VSV_{IND} from an infectious cDNA clone as described earlier (Lawson *et al.,* 1995). BHK-21 cells infected with vTF7–3 were transfected with plasmids encoding L_{IND}, P_{IND}, full-length cDNA clone of VSV_{IND} antigenomic RNA [$pFL_{IND}(+)$], and either wild-type N_{NJ} or N_{IND}. After 48 h posttransfection, the plates were subjected to repeated freeze–thaw treatment, and the lysate was passed through 0.2-μm membrane for removing vaccinia virus. The recombinant VSV was recovered and added to fresh BHK cells for amplification. After 16–24 h p.i. in the presence of araC, the CPE was visualized by staining with crystal violet. As shown in Fig. 4A, two plates that contained the N support plasmids of either serotype showed severe cytopathic effect after 16 h p.i. By 24 h, no attached cells were observed in these two plates, whereas virtually all cells remained intact in control plates that did not have N support plasmid. We also performed plaque assay to determine the titer of the recombinant VSV released into the medium after 24 h p.i. The results of the plaque assay shown in Fig. 4B indicate that the amount of recombinant virus rescued by N_{NJ} protein was comparable with that by N_{IND}. Thus it can be concluded that N_{NJ} is indeed able to encapsidate the genome RNA of VSV_{IND} *in vivo* and can rescue infectious virus from cDNA clone in the presence of L_{IND} and P_{IND}.

Next we tested the abilities of N_{NJ} mutants to support encapsidation or replication of VSV genome RNA *in vivo*. The rescue of infectious VSV_{IND} was carried out as described above from BHK cells expressing L_{IND}, P_{IND}, and the wt or mutant N_{NJ} proteins. The CPE was visualized by crystal violet staining, and the virus recovery from the medium after amplification was measured by plaque assay. The results shown in Fig. 5 indicate that the N mutants that were incapable of encapsidating leader RNA *in vitro* did not rescue infectious virus. It can be seen that no CPE or virus recovery was observed by the mutants CΔK and VEFDK → A_5. On the other hand, the N mutants K → A or NΔ5 that showed encapsidation activity *in vitro* also supported virus recovery (Fig. 5B). From these results we conclude that the N mutants, defective in *in vitro* encapsidation of leader RNA, are unable to

support VSV replication, and more importantly a single Lys residue plays a critical role in this process.

To confirm that all N mutants used in the above experiment were expressed equally, we analyzed the expression of N proteins in transfected cells by Western blotting. Under the transfection conditions, comparable amounts of the mutant N proteins were synthesized in the transfected cells (data not shown).

DISCUSSION

The N protein of VSV is a vital protein that maintains the structural integrity of the genome RNA for transcription and replication by the RNA polymerase complex. The genome RNA is assembled with the N protein in such a way that the N-protein-bound genome RNA template becomes resistant to RNase action, indicating a strong association between the N protein and the genome RNA. This assembly or the encapsidation of the genome RNA with the N protein is a fundamental step of the replication process. The N protein appears to interact with the growing RNA chains during replication and encapsidates both full-length plus- and minus-strand genome RNA from 5′ to 3′ direction. Absence of unassembled full-length plus- or minus-strand VSV RNA in the infected cells suggests that growing RNA chains are the target of N protein interaction. In this study, we have attempted to identify a domain(s) responsible for the encapsidation activity of the N protein. Besides its RNA binding activity, the N protein has also been shown to posses P-binding activity. The presence of N–P complex in VSV-infected cells as well as its role in viral replication has been well established (Peluso and Moyer, 1988; Howard and Wertz, 1989). It is believed that the formation of N–P complex is essential in keeping the N protein in a replication-competent form, which in turn is directly involved in the genome RNA encapsidation process.

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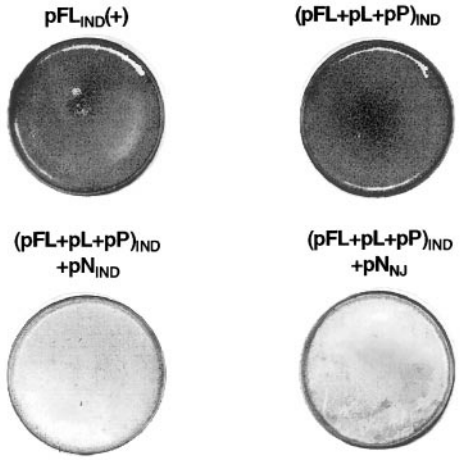
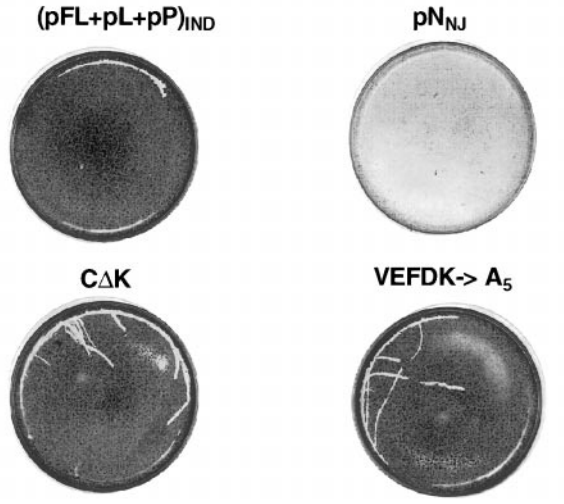


B

Plasmids transfected	No. of plaques at dilution 10^{-4}	10^{-6}
pFL$_{IND}$(+)	0	ND
(pFL+pL+pP)$_{IND}$	0	ND
(pFL+pL+pP)$_{IND}$ +pN$_{IND}$	ND	15
(pFL+pL+pP)$_{IND}$ +pN$_{NJ}$	ND	10

FIG. 4. Rescue of VSV$_{IND}$ from cDNA clone by N$_{NJ}$. (A) BHK cells in 60-mm plates were infected with vTF7–3 at an m.o.i. of 10 and subsequently transfected with 3 μg of pN$_{IND}$ or pN$_{NJ}$, 1 μg of pL, 2 μg of pP, and 5 μg of pFL(+) plasmids. After 48 h posttransfection, the virion particles in the medium were recovered and amplified by infecting fresh BHK cells for 16–24 h. The cytopathic effect (CPE) was visualized by crystal violet staining. (B) After amplification, the recovery of plaque forming units (PFU) in the medium was determined by plaque assay. ND, not determined.

A



B

N$_{NJ}$ proteins	No. of plaques at dilution 10^{-4}	10^{-6}
WT	ND	10
CΔK	0	ND
VEFDK->A$_5$	0	ND
VEF->AAA	0	ND
DK->AA	0	ND
K->D	0	ND
K->A	ND	6
NΔ5	ND	9

FIG. 5. Abilities of mutant N$_{NJ}$ proteins to support replication. *In vivo* replication was carried out as described in Fig. 4. (A) CPE by different N mutants. (B) Recovery of PFU after amplification. ND indicates not determined.

However, the mechanism by which the N–P complex recognizes the nascent RNA chains for encapsidation remains unclear. Clearly the structure–function analysis of the N protein would facilitate understanding the biosynthetic pathways leading to the encapsidation/replication of the virus.

The interesting finding reported here is the critical role of C-terminal penultimate lysine residue of the N protein in the encapsidation process. Deletion of this single amino acid residue resulted in total abrogation of the encapsidation activity (Fig. 3). The lack of RNA encapsidation activity of CΔK mutant N protein appears not to be due to degradation at the C-terminal end by a protease present in the rabbit reticulocyte lysate (or within the cell), because altering K to D or I similarly abrogated the N function. On the other hand, replacing K with R or A did not change its activity. Interestingly, addition of a few extra lysine residues to the penultimate lysine also abolished the encapsidation function of the N protein (data not shown). These results clearly indicate that the penultimate lysine residue plays a vital role in determining the secondary/tertiary structure of the N protein, which facilitates genome RNA replication. Similar *in vitro* RNA encapsidation by rabies virus N protein also has been reported (Yang *et al.,* 1998). In this study, the recombinant N protein binds preferentially to rabies virus leader RNA with 5- to 10-fold more efficiently than the nonleader RNA similar to the observation we made previously for VSV N protein (Das and Banerjee, 1992). Moreover studies with deletion mutants indicate that intact rabies N is required to encapsidate its cognate leader RNA because truncation of a minimum 45 amino acids from either the N or C terminus totally abolished the ability of N to encapsidate rabies leader RNA (Yang *et al.,* 1998). Although the mutational analysis on rabies N was not as extensive as the VSV N protein the basic findings are to some extent similar to our results with respect to the structural requirement of the N protein in RNA encapsidation. Recently, using UV-LASER cross-linking of ^{32}P-labeled leader RNA to rabies virus N protein followed by trypsin digestion, Kouznetzoff *et al.* (1998) have shown that a peptide sequence NH_2 S-P-Y-S-S-N within a 55-aa stretch (position 298–352) as being the primary leader RNA binding site. Interestingly, this amino acid sequence is highly conserved among the lyssa and vesiculoviruses (Kouznetzoff *et al.,* 1998). It is presently unclear whether this RNA binding motif plays a role in maintaining the structural integrity of the N-RNA complex or is involved in the RNA encapsidation process during replication. Mutational analyses of this motif in VSV or rabies virus N protein would reveal interesting results with respect to encapsidation or structural requirement of the motif. In this context, it is noteworthy that the hypervariable C-terminal tail of Sendai virus NP protein was found to be required for template function but not for RNA encapsidation (Curran *et al.,* 1993). This apparently different result from the VSV or rabies systems may indicate the difference in the evolutionary strategies developed between the two families of virus with regard to encapsidation and genome RNA replication.

Because the encapsidation is a prerequisite step to the replication process, we were interested in correlating the *in vitro* encapsidation abilities of the mutant N proteins with the genome RNA replication *in vivo*. Because the mutants we used in the *in vitro* studies were made in N protein of NJ serotype, the replication of VSV_{IND} genome RNA was carried out in the presence of N_{NJ} to first determine whether N_{NJ} could encapsidate VSV_{IND} genome RNA *in vivo*. This approach was based on the fact that the N proteins of both serotypes are highly conserved in terms of amino acid sequence (Banerjee *et al.,* 1984) and on our findings that the N protein of either serotype can encapsidate heterologous leader RNA (Das and Banerjee, 1992, 1993). It was quite apparent that the N protein of NJ serotype could encapsidate VSV_{IND} genome RNA and rescue virion particles from its cDNA clone in the presence of L_{IND} and P_{IND} (Fig. 4). It is worth mentioning that the ability of heterologous viral proteins to support *in vivo* replication of the RNA of defective interfering (DI) particles of two serotypes of VSV was tested previously (Moyer, 1989). In all combinations of heterologous coinfections *in vivo*, DI replication was observed only in the coinfection with the VSV_{IND} DI particle and wild-type VSV_{NJ}, supporting our contention that N_{NJ} is cable of encapsidating VSV_{IND} genome RNA *in vivo*. Thus it seems that the antigenomic or plus-sense RNA of VSV_{IND} synthesized by T7 polymerase is first encapsidated by N_{NJ} protein, and the chimeric template is then replicated to produce genomic or minus-sense RNA enwrapped with N_{NJ}. Clearly the L and P proteins of Indiana serotype were able to replicate the antigenomic RNA enwrapped with N_{NJ} *in vivo*. Because it is known that P_{IND} can form complex with N_{NJ} *in vivo* (Takacs and Banerjee, 1995), it is conceivable that a tripartite complex, L_{IND}-(N_{NJ}-P_{IND}), the presumptive replicase (Das *et al.,* 1997) may carry out the replication of N_{NJ}-enwrapped VSV_{IND} antigenomic RNA. The resultant VSV_{IND} genome RNA enwrapped with N_{NJ} is subsequently transcribed by L_{IND} and P_{IND} to produce Indiana serotype specific mRNAs and the replication of VSV_{IND} genome RNA ensues. Therefore the rate-limiting step in our *in vivo* assay system is the encapsidation of T7 derived VSV_{IND} antigenomic RNA by N_{NJ} protein. Any defect in this crucial step would certainly inhibit the formation of template (N-RNA complex) required for transcription and replication. Thus the N_{NJ} mutants that are defective in encapsidation function should not support replication *in vivo*, and indeed our results shown in Fig. 5 support this contention. The virus-recovery data (Fig. 5) corroborated exactly the *in vitro* results (Fig. 3), confirming again the important role of the C-terminal five amino acids especially the penultimate Lys residue in VSV genome RNA encapsidation/

replication. In rare cases, we have noticed a low level recovery of recombinant virion particles ($\sim 10^3$ pfu/ml) in the absence of a N support plasmid under the assay condition used. It may be due to low-level expression of N protein directly from the T7-derived antigenomic RNA. However, in the presence of N_{NJ} support plasmid, the virus recovery is greatly enhanced ($\sim 10^7$ pfu/ml after amplification), and this observation is fairly consistent in our repeated experiments. To avoid any confusion regarding the background level of virus recovery without N, we performed plaque assay starting from a minimum dilution of 10^{-4} (Figs. 4 and 5) so that we could eliminate the background level if any in that particular set of experiment.

We have demonstrated previously that the C-terminal region of the N protein is also involved in its interaction with the P protein (Takacs *et al.,* 1993; Takacs and Banerjee, 1995). No N–P complexes were formed when C-terminal five amino acid residues were deleted from the N protein (Takacs *et al.,* 1993; Takacs and Banerjee, 1995). Interestingly, the N mutants such as CΔK and DK → AA, which failed to encapsidate the genome RNA, formed complexes with the P protein as wild-type N (Takacs *et al.,* 1993). On the other hand, altering VEF to all alanine residues abrogated both RNA encapsidation and P-binding activities (Takacs *et al.,* 1993). Thus it seems that the DK residues are crucial for RNA encapsidation, whereas VEF are needed for P interaction. The involvement of same C-terminal five amino acid regions in both RNA encapsidation and P-binding properties may have some biological significance. It is possible that in the infected cells, the P protein interacts with the C-terminal domain of the N protein to prevent the latter from binding to cellular or presynthesized viral specific RNAs as observed earlier (Masters and Banerjee, 1988b; Das and Banerjee, 1992; Yang *et al.,* 1998). Additionally, such interaction possibly maintains the N–P complex in a replication competent structure so that it can now recognize nascent 5′ ends of the RNA chains for encapsidation or replication. Deletion of C-terminal five amino acid residues of N results in the complete loss of RNA encapsidation as well as P interacting activity, possibly due to disruption of its secondary or tertiary structure. It is possible that the actual RNA binding domain is located somewhere within the N protein, such as the highly conserved domain found in the middle of the N protein of four vesiculoviruses (Crysler *et al.,* 1990) or the pentapeptide sequence described above (Kouznetzoff *et al.,* 1998). The accessibility of this domain to RNA may be dependent on the C-terminal five amino acids. Mutational analyses in this domain would help establish its real function if any. Alternatively, the synthetic oligopeptides corresponding to this middle domain or the C-terminal end would be useful to study their effect on N–P interaction as well as encapsidation/or replication of viral genome RNA. This approach may help to delineate whether P binding and RNA binding domains are functionally separable. Using a similar approach, Yamashita and Kawai (1990) have demonstrated the vital role of the conserved C-terminal 21 amino acids of VSV P protein in viral transcription process. However, the mechanism of inhibition by the oligopeptide corresponding to the C-terminal 21 amino acid regions remains unknown. Further studies along this line would certainly help us gain insight into the structure and function of the N protein in relation to VSV replication.

MATERIALS AND METHODS

Cells and viruses

Baby hamster (Syrian) kidney cells, BHK-21 (ATCC; CCL 10) were maintained in DMEM (Gibco, Inc.) media supplemented with 7% fetal bovine serum (FBS, Gibco). The Indiana serotype of VSV was purified as described previously (Barik and Banerjee, 1991). Recombinant vaccinia virus (vTF7–3) carrying the bacteriophage T7 RNA polymerase gene (Fuerst *et al.,* 1987) was propagated as described by Li and Pattnaik (1997).

Plasmid constructs

Transcription vectors pN109 (Masters and Banerjee, 1988a), pGEM-N_{IND} (Das and Banerjee, 1992), and pGEM-(+) leader (Das and Banerjee, 1992), which link full-length copies of the coding regions of the N_{NJ}, N_{IND}, and (+)-leader genes of VSV_{NJ}, respectively, to the RNA polymerase promoter of bacteriophage SP6 were used in this studies. To make the mutations at C-terminal end of the N_{NJ} protein, we amplified a 300-nucleotide DNA fragment of the N_{NJ} gene containing various mutations and replaced these PCR-amplified fragments into wt N_{NJ} gene using unique *Xho*I and *Hin*dIII sites of pN109. In contrast, to make the mutations at the N-terminal end, we amplified the entire N_{NJ} gene. In this case, the upstream primer contained an unique *Eco*RI site followed by respective mutations and the complementary sequences to the 5′ end of the N_{NJ} gene, whereas the downstream primer contained the complementary sequence to the 3′ end of the N_{NJ} gene followed by a *Hin*dIII site. The PCR-amplified products were then introduced into pGEM-4 vector at the *Eco*RI and *Hin*dIII sites. PCR conditions were 94°C for 2 min, 50°C for 2 min, and 72°C for 2 min with a 7-min extension at 72°C after 25 cycles. Mutant N_{NJ} sequences were identified by sequencing the amplified products, ensuring that additional mutations were not introduced by PCR.

pVSVFL(+), pBS-N, pBS-P, and pBS-L containing full-length copies of 11 161-nt-long antigenomic VSV RNA sequence, coding regions of N, P, and L, respectively, of VSV_{IND} serotype under T7 promoter in pBluescript SK(+) vector were kindly provided by Dr. John K. Rose, Yale University (Lawson *et al.,* 1995). The wild-type N_{NJ} as well

as all the N_{NJ} mutants made in SP6 transcription vector pGEM4 were subcloned into pBluescript SK(+) vector under the control of T7 promoter for using in *in vivo* replication experiment.

SP6 transcription and *in vitro* translation

Plasmid DNAs were linearized with *Hin*dIII and purified by phenol:chloroform extraction and ethanol precipitation before *in vitro* transcription. SP6 transcription was carried out in a 40-μl reaction containing 40 mM Tris–HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 40 units RNasin, unlabeled rNTPs (500 μM each of ATP and GTP, 100 μM each of CTP and UTP in case of leader RNA while 500 μM each of ATP, CTP, and UTP, 100 $\mu\mu$M GTP, and 500 μM m7GpppG in case of N mRNA), 1 μg of linearized DNA template and 20 units of SP6 RNA polymerase. For labeling RNA, 5 μCi of α-^{32}P UTP (for mRNA synthesis) or 5 μCi each of α-^{32}P UTP and CTP (for leader RNA synthesis) was included in the reaction. Reactions were incubated at 40°C for 1 h. The RNA products were then extracted with phenol:chloroform and twice ethanol precipitated. The incorporation of labeled NTP was determined by binding to Whatman DE-81 filter paper and analyzed by electrophoresis on polyacrylamide gel containing 8 M urea.

The *in vitro* translation of mRNAs was performed using 12 μl rabbit reticulocyte lysate (Promega), 15 units RNasin (Boehringer Mannheim), 7.5 μCi of [^{35}S]-methionine (800 Ci/mmole), and 0.2–0.3 μg of mRNA in a 15-μl reaction mixture. Incubation was carried out for 90 min at 30°C. The translation efficiencies of SP6 mRNAs were ~0.4 μg of protein per μg of N mRNAs of either serotype.

In vitro encapsidation of leader RNA

The *in vitro* encapsidation of VSV leader RNA was carried out in a reaction volume of 30 μl where ~0.1 μg of ^{32}P-labeled leader RNA (~100,000 cpm) was incubated with appropriate mRNAs (0.4–0. 5 μg) at 30°C for 90 min in the presence of 21 μl of rabbit reticulocyte lysate. RNaseA was then added to the reaction at a final concentration of 25 μg/ml and further incubated at 37°C for 30 min. After RNaseA digestion, the reaction volume was increased to 200 μl with TE. RNaseA was inactivated by adding 1 μg of tRNA, 0.5% SDS, and proteinase K at a final concentration of 200 μg/ml. Samples were then extracted with phenol:chloroform and precipitated with ethanol. The protected RNA was analyzed by electrophoresis on a 10% polyacrylamide gel containing 8 M urea.

Rescue of infectious virus

BHK-21 cells in 60-mm plates grown in DMEM plus 7% FBS to ~70% confluency were infected with vTF7–3 at an m.o.i. of 10. After 30 min, plasmids encoding the VSV antigenomic RNA and the N, P, and L proteins were transfected into the cells using lipofectamine (Gibco) according to the manufacturer's protocol. The coding regions for N, P, and L proteins were each expressed in pBluescript SK(+) from the T7 promoter. The amounts of plasmids used in the transfection were 5 μg of pVSV-FL_{IND}(+), 3 μg of pBS-N (either serotype and mutants), 2 μg of pBS-P_{IND}, and 1 μg of pBS-L_{IND}. After 24 h post-transfection in OPTI MEM, the transfection reagent was replaced with fresh DMEM containing 7% FBS and further incubated for 24 h at 37°C. Cells were then scraped from the dish and subjected to freeze-thaw cycles (−70 and 37°C) to release cell-associated virus. Cell debris were removed by centrifugation at 1500 *g* for 10 min. The entire lysate (~4 ml) was passed through a filter (0.2-μm pore size, Millipore) to remove vaccinia virus. The recombinant VSV was pelleted by centrifuging the lysate at 100,000 *g* for 1 h. The pellet was suspended in DMEM without FBS and used for infection of fresh BHK cells in 60-mm plate in the presence of 1-β-D-arabinofuranosylcytosine (araC, 25 μg/ml). The plates were incubated at 37°C for 16–24 h until the cytopathic effect (CPE) was observed. The CPE was visualized by crystal violet staining. The production of virion particles in the medium was determined by plaque assay in the presence of araC.

ACKNOWLEDGMENT

This work was supported by National Institutes of Health Grant AI-26585 (to A.K.B.).

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